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Evolutionary genetics of birds

Comparative molecular evolution in New World warblers and rodents

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THE WOOD WARBLERS (Parulidae) compose a large family of brightly colored, insectivorous songbirds essentially confined to the New World. Evolutionary relationships among parulids, and of parulid species to other Passeriforme birds, historically have been poorly understood[18]. This uncertainty is reflected in the very high percentage (about 50 percent) of monotypic genera within the family. Also, several genera are listed as "*incertae sedis*" in modern classifications[25]. Of the approximately 120 species and 30 genera currently recognized[13,25], about 37 species and 12 genera regularly nest in eastern North America. This study describes electrophoretically assayed protein variation and differentiation in 28 species representing all of these eastern United States genera. One purpose of this study is to elucidate genetic and systematic relationships among wood warblers.

However, the major motivation for this report derives from recent observations in several laboratories that protein evolution in birds appears conservative relative to that of many invertebrate and nonavian vertebrate groups[1,2,9,22,34,41]. By conservative we mean only that at equivalent levels of taxonomic recognition many birds appear to exhibit smaller genetic distances at protein-coding loci than do most other kinds of organisms that have been surveyed. The reasons for this conservative pattern remain unknown. One possibility is that protein evolution is decelerated in birds; the protein "clock" may tick at a slower pace. It is primarily this hypothesis that we wish to evaluate here.

The fossil record for Passeriforme birds is notoriously poor and provides little assistance in determining divergence times[37,39]. For example, only a small handfull of parulid fossils, from Pleistocene and recent deposits, are known. Nonetheless, a bold and compelling reconstruction of the evolutionary history of Parulidae, based upon biogeographic distributions and systematics in relation to Pliocene and Pleistocene history, has been developed[23]. This reconstruction provides a time scale against which to appraise possible rates of protein differentiation in the wood warblers.

We will begin this report by contrasting estimates of genetic divergence among Parulidae with genetic distances previously observed in a group of North American rodents of the subfamily Cricetinae[6,31] (see cover). Cricetine rodents were chosen for this comparison because they were run in our laboratory at the same time using similar electrophoretic conditions and because they typify levels of genetic differentiation characteristic of many nonavian groups.

The authors are affiliated with the Department of Molecular and Population Genetics, University of Georgia, Athens, GA 30602. They are very grateful for the continued help of Mr. Robert Crawford and other workers of the Tall Timbers Research Station in providing most of the bird specimens for their work. They also want to thank Ms. Ida Cantwell for providing the warbler photographs reproduced on the cover. Research was supported by a grant from the Penrose Fund of the American Philosophical Society.

Materials and Methods

Parulid species examined in this study are listed in Table I. To provide a perspective on genetic distances within Parulidae, and to provide "outgroup" comparisons for cladistic analysis, two additional species belonging to Muscicapidae and Vireonidae[25] were included. Most specimens were obtained as fresh "tower-kill" specimens[16] from Tall Timbers Research Station near Tallahassee, Florida, during the spring and fall migrations of 1978-1979. Additional specimens were collected in North Georgia.

Heart, liver, and muscle extracts were subjected to horizontal starch-gel electrophoresis according to standard procedures[8,40], as modified slightly for birds[1]. These same procedures were also employed to examine the cricetine species[31]. The proteins assayed, and the genetic loci encoding their production, are listed in Table II. A total of 16 proteins encoded by 26 loci was examined in the Parulidae although not all species could be scored for all loci.

Barrowclough and Corbin[9] have previously assayed electrophoretic mobilities of proteins encoded by about 30 loci in 15 species and 9 genera of Parulidae. Their overall results (levels of heterozygosity and genetic distance) closely match our own. Our study represents an extension and amplification of several suggestions originally made by Barrowclough and Corbin. Therefore, for sake of completeness we have also provided, where appropriate, a summary of results of that study.

Electromorphs were assigned numerical values determined by mobility relative to the common electromorph at each locus in *Geothlypis trichas* (Parulidae) or *Peromyscus polionotus* (Cricetinae). In all cases, electromorphs of similar mobility were compared side-by-side on the same gels. Genetic distances between all pairs of species were calculated using Nei's[27] formulas. Distances in the species' matrix were phenetically clustered using the unweighted pair-group algorithm with arithmetic means (UPGMA)[42].

Results

Genetic variation within parulid species

For each species, direct counts were made of the proportion of individuals heterozygous per locus. When averaged across

18–24 assayed loci, the resulting heterozygosity (H) values ranged from a low of 0.004 (*Dendroica magnolia*) to a high of 0.128 (*Seiurus noveboracensis*) (Table I). The mean heterozygosity for all 28 parulid species was $\overline{H} = 0.043$ (nearly identical to the value of $\overline{H} = 0.046$ reported by Barrowclough and Corbin[9] and 15 warbler species). As is usual in such multi-locus studies, some loci contribute much more to $\overline{H}$ values than do others. In the Parulidae, *NP, PGI, PGD, GPD, PEP-1,2,* and *IDH-1* frequently appear polymorphic, while *LDH-1,2, MDH-1,2, GOT-2, IDH-2, CK-4, PT-1, PT-2, ADKIN,* and *HB* appear monomorphic within most or all species. Other assayed loci are intermediate in contribution to heterozygosity. Considering the small sample sizes of many species, and the large standard errors of H across loci (Table I), it would be unwise to attach too much biological significance to differences in heterozygosities among warbler species. Nonetheless, mean heterozygosity for the family is very close to mean values previously reported for other bird families[1,2,9], and for most vertebrates[29,33], including the cricetine rodents[5,6]. Clearly, parulids are not poor in within-species genetic variation.

Genetic divergence in Parulidae versus Cricetinae

Space does not permit a complete listing of electromorph frequencies in parulids. The summary matrix of overall genetic distances among species alone contains 378 values. The majority of these values are presented in Tables III–V. Many distances are very small and not meaningfully different from one another. If we temporarily exclude the yellow-breasted chat (*Icteria virens*) from consideration, genetic distances among the remaining 27 warbler species in 11 genera ranged from 0.000 (*Dendroica magnolia* versus *D. pensylvanica* and *D. coronata* versus *D. discolor*) to 0.391 (*Mniotilta varia* versus *Dendroica petechia*). Mean genetic distance among species of a genus (74 comparisons) was $\overline{D} = 0.056$, and between species of different genera (277 comparisons) was $\overline{D} = 0.175$ (Table VI). Many pairs of species (72 of 351 or 20.5 percent of all comparisons) were remarkably similar in electromorph composition at all assayed loci, exhibiting D values less than 0.05, and an additional 47 pairs of species (13.4 percent) exhibited $0.05 \leq D \leq 0.10$. Avise et al.[4] have previously summarized an extensive literature of electrophoretically assayed multi-locus genetic distances among congeners in a variety of non-avian vertebrate and invertebrate groups. Of 615 species comparisons surveyed, at most only 6 percent yielded estimates of $D \leq 0.10$, and only a few showed $D \leq 0.05$; and some of these cases involved comparisons among populations whose species status could be questioned. All of the warblers examined are good biological species (see beyond). The low level of genetic divergence among warbler species, when viewed within the context of genetic distances at comparable loci in non-avian organisms, is striking.

In order to further document and dramatize the conservative

Table I. Species examined in this study as classified by Morony et al.[25]; heterozygosities were determined by direct counts of proportions of individuals heterozygous per locus, averaged across 18–26 assayed loci

Species	English name	Sample size	$H \pm$ SE
Parulidae			
1) *Mniotilta varia*	Black-and-white warbler	6	0.026 ± 0.018
2) *Vermivora chrysoptera*	Golden-winged warbler	2	0.048 ± 0.033
3) *V. pinus*	Blue-winged warbler	1	0.095 ± 0.066
4) *V. peregrina*	Tennessee warbler	24	0.069 ± 0.035
5) *V. celata*	Orange-crowned warbler	12	0.040 ± 0.027
6) *Parula americana*	Northern parula warbler	6	0.030 ± 0.018
7) *Dendroica petechia*	Yellow warbler	2	0.068 ± 0.050
8) *D. pensylvanica*	Chestnut-sided warbler	10	0.014 ± 0.008
9) *D. caerulescens*	Black-throated blue warbler	6	0.040 ± 0.022
10) *D. pinus*	Pine warbler	2	0.050 ± 0.034
11) *D. virens*	Black-throated green warbler	2	0.024 ± 0.024
12) *D. discolor*	Prairie warbler	8	0.012 ± 0.008
13) *D. tigrina*	Cape may warbler	6	0.033 ± 0.025
14) *D. fusca*	Blackburnian warbler	8	0.036 ± 0.030
15) *D. magnolia*	Magnolia warbler	12	0.004 ± 0.004
16) *D. coronata*	Yellow-rumped warbler	10	0.005 ± 0.005
17) *D. palmarum*	Palm warbler	33	0.031 ± 0.013
18) *D. castanea*	Bay-breasted warbler	12	0.024 ± 0.014
19) *Setophaga ruticilla*	American redstart	16	0.031 ± 0.011
20) *Seiurus aurocapillus*	Ovenbird	29	0.061 ± 0.020
21) *S. noveboracensis*	Northern waterthrush	7	0.128 ± 0.043
22) *Limnothlypis swainsonii*	Swainson's warbler	2	0.100 ± 0.058
23) *Helmitheros vermivorus*	Worm-eating warbler	2	0.055 ± 0.038
24) *Protonotaria citrea*	Prothonotary warbler	2	0.050 ± 0.050
25) *Geothlypis trichas*	Common yellowthroat	32	0.037 ± 0.012
26) *G. formosa*	Kentucky warbler	16	0.056 ± 0.026
27) *Wilsonia citrina*	Hooded warbler	6	0.015 ± 0.011
28) *Icteria virens*	Yellow-breasted chat	5	0.020 ± 0.014
Muscicapidae			
29) *Catharus ustulatus*	Swainson's thrush	5	0.032 ± 0.023
Vireonidae			
30) *Vireo olivaceus*	Red-eyed vireo	5	0.022 ± 0.015
	Totals	289	0.042

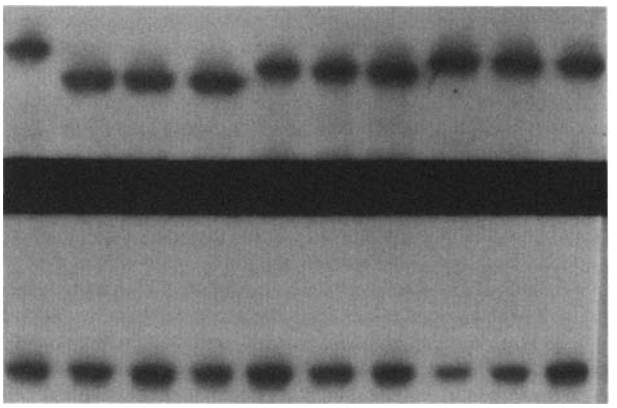

FIGURE 2—Electrophoretic mobilities of the supernatant form of malate dehydrogenase (*MDH-1*) in representative Parulidae and Cricetinae. *Above*, left to right, Cricetinae: one *Ochrotomys nuttalli* (allele *MDH-1*^{100}); three *Reithrodontomys fulvescens* (*MDH-1*^{70}); three *Sigmodon hispidus* (*MDH-1*^{90}); three *Peromyscus leucopus* (*MDH-1*^{100}). Below, left to right, Parulidae: one *Icteria virens*; three *Geothlypis trichas*; three *Vermivora peregrina*; three *Dendroica magnolia*. The parulids, as well as the majority of species in over 20 avian orders, exhibit the same electromorph at *MDH-1*.

nature of protein evolution in Parulidae, we can directly contrast results with those previously reported on a group of rodents of even lower taxonomic rank, the Cricetinae[6,31]. Among 128 comparisons of rodent congeners, $\bar{D} = 0.40$, almost eight times the mean value for Parulidae. Among 83 comparisons between genera of rodents, $\bar{D} = 1.256$, again more than seven times the corresponding value for Parulidae (Table VI). Yet the results for the cricetines are quite typical for many other vertebrate and invertebrate groups[3,7]. The difference between patterns of genetic divergence in the Parulidae versus representative Cricetinae can be easily visualized by comparing dendrograms for both groups against a common scale (Figure 1). For example, 11 species of *Dendroica* (plus representatives of three other warbler genera) form a cluster at a mean level of genetic distance slightly less than that observed between *Peromyscus polionotus* and *P. maniculatus*, two of the most closely related of all *Peromyscus* species. Also, 27 warbler species representing 11 genera form a cluster at a level of genetic distance considerably less than that exhibited by several species of *Peromyscus*.

In both Parulidae and Cricetinae (as well as in most other organisms surveyed) there is considerable variance across loci in contribution to overall genetic distance. For example, a total of 18 electromorphs at *PGD* were observed in our samples of 28 parulid species, and at least six of these represented the predominant electromorph in one or more species. At the opposite extreme, the locus encoding the supernatant form of malate dehydrogenase (*MDH-1*) appears identically monomorphic not only in all the Parulidae (Figure 2), but in the great majority of species belonging to over 20 avian orders[19]. On the basis of banding patterns and tissue specificities, a total of nine assayed loci can reliably be considered homologous across Parulidae and Cricetinae. For these individual loci, estimated levels of allelic diversity in the New World warblers and rodents are presented in Table VII. Despite the fact that twice as many species of parulids were examined, loci consistently demonstrated greater allelic diversity within Cricetinae. On the average, the locus-specific level of allelic diversity within Parulidae appeared about one-fifth as high as that within our more limited sample of rodents.

Systematics of Parulidae

The very low level of genetic divergence among 27 parulids (excluding *Icteria*) inhibits attempts to unambiguously define genetic relationships within the assemblage although we can be very confident about the close evolutionary ties of all species. We had originally intended to include a qualitative cladistic

Table II. Proteins assayed in various studies of Parulidae and Cricetinae

Protein	Locus abbrev.*	Tissue source†	Reference: Present study	9	31	5,6
Acid phosphatase	*ACP*	L		√		
Adenylate kinase	*ADKIN*	L	√			
Alcohol dehydrogenase	*ADH*	L				√
Creatine kinase	*CK-1,3*	H	√		√	
	CK-4	M	√	√		
Esterase	*EST-1,2*	B		√	√	√
	EST-3	L		√		
	EST-4	H	√			
Glucose-6-phosphate dehydrogenase	*G-6-P*	H				√
Glutamate dehydrogenase	*GDH*	M		√		
Glutamate-oxaloacetate transaminase	*GOT-1,2*	M,H	√	√	√	√
α-Glycerophosphate dehydrogenase	*GPD*	M	√	√		√
Hemoglobin	*HB*	B	√			√
Indophenol oxidase	*IPO*	H			√	√
Isocitrate dehydrogenase	*IDH-1,2*	L,H	√	√	√	√
Lactate dehydrogenase	*LDH-1,2*	H,M	√	√	√	√
Malate dehydrogenase	*MDH-1,2*	H	√	√	√	√
Malic enzyme	*ME*	H		√	√	
Mannose phosphate isomerase	*MPI-1,2,3*	H		√		
Nucleoside phosphorylase‡	*NP*	L	√			
Peptidase	*PEP-1,2,3*	L	√	√		
6-Phosphogluconate dehydrogenase	*PGD*	L,H	√	√	√	√
Phosphoglucomutase	*PGM-1,2,3*	M,L	√	√		√
Phosphoglucose isomerase	*PGI*	L	√			√
Sorbitol dehydrogenase	*SDH*	M,H		√	√	√
Superoxide dismutase	*SOD*	M		√		
General proteins	*PT-1*	M		√		
	PT-2-3	B,H	√	√		√
	PT-4-7	B				√

* Does not necessarily imply homology across studies; abbreviations have in some cases been changed from those in the original papers
† L = liver; H = heart; M = muscle; B = blood
‡ Was not included in estimates of genetic distance, due to scoring difficulties across species

analysis[1,31] of the data by distinguishing plesiomorphic (ancestral) from apomorphic (derived) alleles on the basis of "outgroup" comparisons with *Catharus ustulatus* and *Vireo olivaceus*. This proved to be infeasible, however, due to the nearly complete absence of electromorphs that could be identified as synapomorphic (shared-derived). Therefore, we will very briefly discuss results of a phenetic data analysis, reflected in the overall genetic distance values and summarized in Figure 1. We doubt that further manipulation of quantitative values in a distance matrix[35], without the addition of information from greater numbers of loci, will provide significant additional insights into the systematics of parulid species. The following sketch of the systematic implications of our genetic data is not intended to be definitive, but does identify problem areas for further study.

Dendroica. This is the largest genus of wood warblers, containing 27 species of which we have examined 12. Mean genetic distance among these species is extremely low, $\bar{D}$ = 0.043 ± 0.006. With the following few exceptions, common electromorphs were shared between *Dendroica* species at all loci: *D. petechia* exhibited alleles at *GOT-1, PGI,* and *PEP-1* which were rarely observed in other *Dendroica; D. castanea* was monomorphic for a unique allele at *ADKIN*. With the exception of *D. petechia*, which proved to be the most genetically distinctive species, all members of *Dendroica* essentially cluster together in the dendrogram (Figure 1).

Geothlypis. The two species examined (*formosa* and *trichas*) were until recently[25] placed in distinct genera (*Oporornis* and *Geothlypis*, respectively). *Formosa* and *trichas* are nearly identical in allelic composition at all assayed loci ($\bar{D}$ = 0.024) and cluster together in the dendrogram.

Protonotaria. This monotypic genus, containing only *P. citrea*, is very similar to *Geothlypis* in allelic composition at all 21 loci compared ($\bar{D}$ = 0.082), and clusters with *Geothlypis* in the phenogram. Nonetheless, this larger cluster exhibits no electromorphs peculiar to this assemblage alone. *Protonotaria* is placed adjacent to *Geothlypis* in most current check lists that attempt to index systematic position[25].

Seiurus. Somewhat surprisingly, given the very small $\bar{D}$'s within other warbler genera, *Seiurus aurocapillus* and *S. noveboracensis* showed little overlap in electromorph frequencies at *GPD, ADKIN, PEP-1, EST-1,* and *CK-1*. Mean distance was $\bar{D}$ = 0.248, and the species appear in distinct clusters of the phenogram. Barrowclough and Corbin[9] previously described rather large allele frequency differences between these two species at about five of 30 loci, although the species did occupy the same branch of a phylogenetic network, distinct from seven other parulids. In both studies, *noveboracensis* appears phenetically closer to several species of *Dendroica* than does *aurocapillus*.

Vermivora. We observed four species of this genus to fall into two distinct genetic groupings: *V. celata-V. peregrina*, and *V. chrysoptera-V. pinus* (Figure 1). Species within each of these two pairs are very similar genetically ($\bar{D}$'s = 0.003 and 0.012, respectively), but the two pairs are essentially distinct from one another in allelic composition at *PT-2, PEP-1, EST,* and *CK-1* ($\bar{D}$ = 0.264 between pairs).

Mniotilta. The Black-and-white warbler (*M. varia*) is genetically relatively distinct from the other warbler species ($\bar{D}$ = 0.283). It clusters most closely to *Vermivora chrysoptera-V. pinus* ($\bar{D}$ = 0.178), and shares with these two species common electromorphs at *PT-2* and *CK-1*, which were only rarely observed in other warblers.

Wilsonia. At 24 assayed loci, the hooded warbler (*W. citrina*) exhibited only one diagnostic locus (*EST-4*) by which it could be distinguished from several *Dendroica* species scored. It clusters with the *Dendroica* assemblage in Figure 1.

Setophaga. The American redstart (*S. ruticilla*) differed from most of the *Dendroica* species in allelic composition at *CK-3* and *EST-4*, but was otherwise genetically similar to them ($\bar{D}$ = 0.094). In the dendrogram, it falls just outside of the main *Dendroica* cluster.

Parula, Limnothlypis, and *Helmitheros*. Two of these genera are monotypic and the third (*Parula*) contains only two species. Species in these genera are among the more genetically distinct of the warblers examined, and exhibit no particular tendency to cluster with other species groupings or with one another (Figure 1). Most of their distinctness derives from possession of unique (autapomorphic) alleles, or alleles that are rare in other warblers, at the following loci: *GPD, PEP-2*

Table IV. Matrix of genetic distances (Nei's *D*) between congeneric species within each of the warbler genera *Vermivora*, *Seiurus*, and *Geothlypis*

Species	(A)	(B)	(C)	(D)
A) *V. peregrina*	—	0.003	0.249	0.274
B) *V. celata*		—	0.254	0.279
C) *V. chrysoptera*			—	0.012
D) *V. pinus*				—
S. aurocapillus vs. *S. noveboracensis*			0.248	
G. trichas vs. *G. formosa*			0.024	

Table III. Matrix of genetic distances (Nei's *D*) between species of the warbler genus *Dendroica*

Species	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)
A) *D. palmarum*	—	0.003	0.049	0.026	0.029	0.025	0.005	0.025	0.120	0.026	0.007	0.002
B) *D. discolor*		—	0.046	0.023	0.026	0.023	0.003	0.029	0.116	0.024	0.004	0.000
C) *D. castanea*			—	0.071	0.075	0.071	0.049	0.078	0.165	0.073	0.049	0.046
D) *D. pensylvanica*				—	0.003	0.000	0.026	0.006	0.144	0.002	0.027	0.023
E) *D. tigrina*					—	0.003	0.029	0.009	0.142	0.005	0.032	0.026
F) *D. magnolia*						—	0.026	0.006	0.147	0.002	0.028	0.023
G) *D. virens*							—	0.031	0.119	0.027	0.008	0.003
H) *D. pinus*								—	0.156	0.005	0.034	0.028
I) *D. petechia*									—	0.141	0.108	0.113
J) *D. caerulescens*										—	0.030	0.024
K) *D. fusca*											—	0.005
L) *D. coronata*												—

(*Parula*); *PGI, PGD, IDH-1* (*Limnothlypis*); *PGD, GPD, PT-2* (*Helmitheros*).

Icteria. The yellow-breasted chat (*Icteria virens*) is a highly aberrant warbler with respect to size, song, and general behavior[17]. It has been classified by various authors with manakins (Pipridae), vireos (Vireonidae), and honeycreepers (Coerebidae)[10], but is now generally classified as an unusual parulid with uncertain generic affinities. By "bird" standards, the yellow-breasted chat appears very distinct genetically from the other Parulidae examined: $\overline{D} = 0.483$. It possesses at several loci (*PGM-2, PGD, GPD, IDH-1, HB, PEP-2, EST-4*) common electromorphs seldom or never observed in the other warblers. Nonetheless, it appears more similar phenetically to Parulidae than do *Vireo olivaceus* or *Catharus ustulatus* (Figure 1). It is also as similar to the Parulidae as are two of the most closely related genera of cricetines (*Peromyscus* and *Onychomys*) to one another.

Catharus ustulatus and *Vireo olivaceus.* These species (belonging to Muscicapidae and Vireonidae, respectively) were included in this study as outgroup taxa. Both are genetically quite distinct from all Parulidae examined, although they fall within the range of genetic distances observed among cricetine genera (Figure 1, Table III). We have recently found *Vireo olivaceus* to be a genetically divergent species among several Vireonidae assayed (unpublished data). For this reason, additional comparisons of Parulidae with Vireonidae are required before we can conclude that the vireos are phenetically more distinct from the wood warblers than are thrushes.

Discussion

The protein clock in Parulidae

The salient result of this study is the extreme conservatism in level of protein divergence among a large number of species and genera of Parulidae. Most of the intragenus distances in Parulidae (and in several other avian families) fall within the

Table VII. Locus-specific levels of allelic diversity (*S*) for homologous genes in Parulidae (12 genera, 28 species) and Cricetinae (9 genera, 14 species)

Locus	*S** Parulidae	*S** Cricetinae
1) *GOT-1*	0.206	1.177
2) *GOT-2*	0.163	1.088
3) *IDH-1*	0.630	1.346
4) *IDH-2*	0.030	1.214
5) *LDH-1*	0.026	1.663
6) *LDH-2*	0.000	0.970
7) *MDH-1*	0.000	0.654
8) *MDH-2*	0.022	0.598
9) *PGD*	1.088	2.761
Totals	0.240	1.274

* $S = -\Sigma p_i \ln p_i$, where p_i is the frequency of the ith allele in the family or subfamily, determined by weighting equally each species

Table V. Matrix of mean genetic distances (Nei's *D*) between species in different genera of Parulidae

Genus	(# species examined)	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)
A)	*Geothlypis* (2)	—	0.141	0.182	0.116	0.184	0.146	0.174	0.215	0.340	0.211	0.082	0.440
B)	*Seiurus* (2)		—	0.234	0.141	0.198	0.155	0.273	0.233	0.328	0.200	0.154	0.518
C)	*Vermivora* (4)			—	0.178	0.244	0.194	0.234	0.252	0.221	0.221	0.199	0.513
D)	*Dendroica* (12)				—	0.094	0.052	0.222	0.158	0.262	0.152	0.116	0.463
E)	*Setophaga* (1)					—	0.088	0.305	0.240	0.345	0.201	0.158	0.530
F)	*Wilsonia* (1)						—	0.254	0.190	0.287	0.152	0.112	0.467
G)	*Helmitheros* (1)							—	0.331	0.294	0.266	0.187	0.484
H)	*Limnothlypis* (1)								—	0.351	0.270	0.222	0.501
I)	*Mniotilta* (1)									—	0.309	0.332	0.693
J)	*Parula* (1)										—	0.175	0.500
K)	*Protonotaria* (1)											—	0.350
L)	*Icteria* (1)												—

Table VI. Genetic distance between species of Parulidae and Cricetinae at various levels of taxonomic divergence

	Comparison	comparisons	mean	(range)	Data source
Parulidae	(New World warblers)				
	Within genera	74	0.056	(0.000-0.279)	present study
		10	0.100	—	9
	Between genera (excluding *Icteria*)	277	0.175	(0.011-0.391)	present study
		95	0.179	—	9
	Icteria versus other genera	27	0.483	(0.350-0.693)	present study
	Parulidae versus Vireonidae and Muscicapidae	56	0.998	(0.679-1.663)	present study
Cricetinae	(New World rodents)				
	Within genera	8	0.436	(0.114-0.909)	31
		120	0.393	(0.002-1.002)	5,6
	Between genera	83	1.256	(0.398-2.238)	31

range characteristic of conspecific populations in the cricetine rodents and in many other vertebrates and invertebrates; and intergenus distances in Parulidae are characteristic of values for closely related congeners elsewhere. It is well known that the electrophoretic methods employed underestimate true genetic distances at surveyed loci[11,14,15], but there is no compelling reason to believe that the underestimate is greater for birds than for other organisms assayed with these same techniques. Furthermore, heterozygosity estimates in Parulidae (Table 1) are very similar to values reported in most other vertebrates. Thus our techniques are at least sufficiently sensitive to uncover considerable within-population genetic variation.

One of the most significant results of research in molecular evolution is the finding that the decay of protein similarity among reproductively isolated populations is progressive and correlated with period of time since separation of gene pools[20,45]. Recent efforts have focused on attempts to calibrate the "protein clock" against absolute time scales ultimately derived from fossil evidence. The calibration is complicated by the observation that different proteins evolve at different rates, a consideration taken into account in recent approaches[38]. We have applied calibrations suggested in several papers to estimate mean absolute divergence times from genetic distances observed in Parulidae, under the assumption that the protein clock for parulids is synchronized with those for other groups (primarily mammals, reptiles, amphibians, and *Drosophila*[12,26,27,38]) upon which the clocks were originally timed. Results are presented in Table VIII.

It is evident that estimated divergence times vary tremendously depending on the calibration employed. For example, estimated mean divergence times for congeneric species of Parulidae range nearly 20-fold from a low of 100,000 years to a high of 1.8 million years. Nonetheless, with the exception of method B of Sarich (see below), all clocks date the separation of parulid species, and most parulid genera, to the middle or late Pleistocene.

Mengel[23] has presented a detailed and persuasive reconstruction of possible divergence times for many species of wood warblers in North America, based on zoogeographic and systematic considerations. According to the scenario, Parulidae originated in the North American tropics probably in the late Oligocene or early Miocene (ca 25 million years ago), and had diversified considerably by early Pliocene (ca 5 million years ago) when many members of the group were adapted to the deciduous Arcto-tertiary forests of southeastern North America. During the Pleistocene, for which the most detailed reconstruction is possible, radiation was also extensive, but produced primarily east-west differentiates at the semispecies or closely related "species-group" level. With a few exceptions (*Vermivora chrysoptera-V. pinus; V. celata-V. peregrina;* possibly *Dendroica castanea-D. tigrina-D. pensylvanica*[23]), all of the species we examined belong to distinct species complexes, or to different genera. If Mengel's reconstruction is correct, the great majority of comparisons we have made involve warblers that last shared a common ancestor more than two million years ago.

This creates a dilemma: either the time scale of Mengel's scenario is incorrect, or the apparent rate of protein divergence is slower in Parulidae than in many nonavian vertebrates. Only the protein clock proposed by Sarich[38] (method B, Table VIII) provides divergence times easily accordant with Mengel's proposals. This protein clock was calibrated under the assumption that all assayed loci belong to the "slowly evolving" class. Yet we have included in our study two general proteins, hemoglobin, two peptidases, and an esterase, proteins which are typically found to be "rapidly evolving"[38]. If this sluggish clock proves appropriate for Parulidae, equally important questions would remain: 1) why do so many protein loci appear to evolve slowly? and 2) is the slowdown a genome-wide phenomenon?

We feel that the sum of all available information is not yet sufficient to flatly resolve the dilemma posed above. For example, some authors, without specifically referring to Parulidae, believe that most living bird species arose in the Pleistocene[24]. On the other hand, Prager and Wilson[35] have provided immunological evidence that albumin and transferrin "have evolved about three times as slowly in birds as in other vertebrates." Therefore, the evidence for a possible slowdown of protein evolution in birds is at least sufficiently compelling to warrant issue of a general admonition: extreme care should be exercised in basing divergence times in poorly fossilized groups *solely* on protein clocks calibrated for very different kinds of organisms.

Biological divergence in Parulidae

It might be argued that protein evolution in Parulidae appears conservative because the group has been taxonomically "oversplit" relative to nonavian vertebrates. This may be true at the generic level; as mentioned in the introduction, Parulidae contains an unusually large proportion of monotypic genera. But parulids are definitely not oversplit at the level of species. All taxa assayed in this study represent unambiguous biological

Table VIII. Approximate mean divergence times (in millions of years) of parulid warblers, determined from various protein clocks calibrated for nonavian vertebrates and invertebrates; validity of the time estimates depends upon the assumption that the protein clock for parulids is synchronized with those for other organisms

Comparison	Mean genetic distance $\overline{D}$	Reference for protein clock (26) $(t = \overline{D}/2cn_t\lambda a)$*	(28) $t = 5 \times 10^6\overline{D}$	(12) $t = 2 \times 10^6\overline{D}$	(38)† A	(38)† B
1) within genera	0.06	0.1	0.3	0.1	0.5	1.8
2) between genera	0.18	0.2	0.9	0.4	1.7	5.4
3) *Icteria* versus other Parulidae	0.48	0.5	2.4	1.0	6.7	14.4

* t, time; c, proportion amino acid substitutions electrophoretically detectable, assumed equal to 0.30; n_t, mean number amino acids per protein, assumed equal to 800; λa, rate of amino acid substitutions per polypeptide per site per year, assumed equal to 2.1×10^{-9} (26)

† A: assumes three-fourths of loci "slowly evolving"; B: assumes all loci "slowly evolving"

species. Virtually all of the species are strikingly different in breeding plumage (different species exhibit predominant reds, yellows, blues, greens, browns, black, or white, variously streaked and patterned[32]), song (ranging from buzzes and flat trills to elaborate warbles), behavior (highly arboreal to predominantly ground-feeding), ecology, and general life history[10,21]. Hybridization is rare, particularly among sympatric congeners[30].

Judging by the striking biological differences, at least some part of the genome must be rapidly evolving in the Parulidae. Wilson and his colleagues[43-46] have argued that regulatory genes are primarily responsible for reproductive incompatibilities and morphological divergence. In the warblers, morphological and life history differences reinforce reproductive isolation, which is largely prezygotic. Even taxonomically distant warblers retain the physiological capacity to produce viable hybrids[30]. Elsewhere, it has been suggested that the slow evolutionary rate of loss of physiological hybridization potential evidences slow regulatory evolution in birds[36]. However, in warblers the loss of hybridization potential evolves slowly despite what appears to be a rapid pace of morphological evolution. These considerations underscore our general ignorance about the genetic processes responsible for organismal evolution.

Summary

The American wood warblers (Parulidae) exhibit considerable diversity in breeding plumage coloration, song, behavior, ecology, and general life history. Nonetheless, a comparison of 28 species representing 12 genera discloses a very conservative pattern of protein differentiation as gauged by standard electrophoretic procedures. We define conservative to mean simply that at equivalent levels of the taxonomic hierarchy, parulids exhibit far smaller genetic distances than do most other organisms surveyed. This observation is documented and dramatized by comparison of results with those obtained in the same laboratory, using similar electrophoretic conditions, on a group of American rodents of even lower taxonomic rank—the Cricetinae. Our study represents an extension and elaboration of similar findings, earlier reported by Barrowclough and Corbin, on parulid warblers.

One possible explanation for this conservative pattern is that warbler speciations have been very recent. However, estimated divergence times for Parulidae, read from protein clocks calibrated for nonavian vertebrates and invertebrates, are much lower than estimates derived from a prevailing view of parulid zoogeography and evolution. It appears likely that protein evolution is decelerated in the wood warblers. If protein clocks generally prove to exhibit organism dependent calibration, their usefulness in determining absolute divergence times for species with a poor fossil record will be compromised.

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